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Multivariate Analysis of Integrated and Full-Resolution ^1H -NMR Spectral Data from Complex Pharmaceutical Preparations: St. John's Wort

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Abstract

Commercial herbal preparations are typically very complex mixtures and the relationship between content of various constituents and pharmacological action of the formulation is usually unclear. Such formulations are nevertheless standardized using a single marker constituent or a group of closely related constituents, which provides no information about other abundant constituents present in the extract. In this study, principal component analysis of 600 MHz ^1H -NMR spectra of extracts of commercial formulations of St. John's wort (*Hypericum perforatum*), acquired in methanol- d_4 and DMSO- d_6 , was shown to be able to discriminate between various preparations according to their global composition, including differentiation between various batches from the same supplier, while no clustering into classes of tablets and capsules was observed. This suggests that the plant extract variability rather than the manufacturing process

accounts for the data clustering. Major variations in the content of flavonoids, recently linked to the antidepressant activity of St. John's wort extracts, were detected. Use of two NMR solvents provided complementary data sets, allowing assessment of various aspects of sample composition from separate PCA models. Both integrated (about 200 variables) and full-resolution NMR data (about 30 000 variables) have been used. The latter approach, applied for the first time in analysis of a herbal preparation, provided via loading plots more precise information about constituents responsible for data clustering, and may be generally preferable for PCA analysis of NMR data of plant extracts and herbal medicines.

Key words

St. John's wort · NMR spectroscopy · pattern recognition · principal component analysis · data reduction · quality control

Introduction

Extracts of St. John's wort (*Hypericum perforatum*) are known to contain several different classes of compounds, but standardization of commercial extracts is only based on the total content of hypericins, as required by the European Pharmacopoeia monograph [1]. This implies that the extracts may be variable with respect to the remaining constituents. Variability between and

within various batches of commercial preparations may result from different methods of cultivation and extraction, environmental factors, time of harvesting, storage conditions, and natural intraspecific variation in St. John's wort [2].

St. John's wort has traditionally been used for the treatment of excitability, neuralgia, fibrositis, sciatica, menopausal neurosis, anxiety, depression, as a nerve tonic, a diuretic agent, an antima-

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larial agent, and in topical preparations for treatment of wounds [3], [4]. Today, the extracts are widely sold as natural remedies for the treatment of mild to moderate depression. A recent study has shown that extracts of St. John's wort are at least as effective in ameliorating symptoms of moderate to severe depression as paroxetine [5].

The mechanism underlying the antidepressant activity of St. John's wort is still not fully understood, but several compounds belonging to different structural classes and having different mechanisms of action seem to be responsible for the observed activity. Thus, flavonoids, hyperforins, and hypericins appear to contribute to the antidepressant activity of St. John's wort [4]. Because of these uncertainties, rapid and efficient analytical methods providing comprehensive information about as many extract constituents as possible would be highly valuable. One such method is multivariate analysis of ^1H -NMR data [6], [7], [8]. High-resolution ^1H -NMR spectra contain signals from all hydrogen-containing compounds present in the extract and may, in the case of complex mixtures, contain thousands of recognizable and diagnostic resonances. Such data can be analyzed using different chemometric methods, since the spectra are far too complex to be interpreted visually. ^1H -NMR spectroscopy in combination with multivariate techniques has previously been applied to plant extracts [9], [10], [11], [12], [13], [14], but usually in the context of biological variability of cultivars or species differentiation, and only in one case in relation to a commercial formulation [15], where the manufacturing process may influence the product composition. Thus, the metabolic fingerprints of commercial formulations of feverfew obtained by ^1H -NMR spectroscopy have been shown to be superior to those obtained by HPLC in terms of discrimination between samples [15]. Furthermore, NMR spectroscopy has the advantage of being non-destructive and intrinsically more information-rich than other spectroscopic techniques with respect to molecular structure elucidation.

In this study, multivariate data analysis of the ^1H -NMR spectra of a number of commercial St. John's wort products has been performed in order to compare samples that have been subjected to the same standardization procedure.

Materials and Methods

Sample preparation

Ten different samples belonging to nine brands of St. John's wort preparations, currently or previously available in Denmark, were obtained from commercial suppliers. For one of the brands, two different batches were obtained. Four preparations were formulated as tablets (preparations 1–4), and the remaining ones as capsules (preparations 5–10). An amount of powder corresponding to 300 mg of plant extract was extracted with 10 mL of MeOH/pyridine (6:4) by sonication for 45 min. The extracts were centrifuged at 18 000 g for 8 min, the powder material was re-extracted as above, and the combined extracts were evaporated, removing residues of the extraction solvents by evaporation with toluene. Light was excluded as much as possible during all operations to minimize degradation of hyperforins.

NMR spectroscopy

Samples of the extracts (10 mg) were dissolved in 750 μL of DMSO- d_6 or in 800 μL of methanol- d_4 ; in the latter case, 750 μL aliquots were transferred into 5 mm NMR tubes after centrifugation for 5 min at 18 000 g in order to remove small amounts of insoluble material. Each sample was prepared in triplicate. ^1H -NMR spectra for multivariate analysis were recorded at 298 K on a Bruker Avance 600 spectrometer (Bruker BioSpin; Karlsruhe, Germany) equipped with a 5 mm inverse probe-head, using standard Bruker library pulse sequences. For each sample, 128 transients were collected as 64 k data points with a spectral width of 20 ppm, using 30° pulses and inter-pulse delay of 3.72 s in order to obtain fully relaxed spectra. The data were Fourier-transformed to 128 k data points using line broadening of 0.3 Hz. The spectra were referenced to internal TMS.

For assignment of secondary metabolites, 1 mL of D_2O was added to 20 mg of extract, the sample was freeze-dried, and the residue was dissolved in DMSO- d_6 or methanol- d_4 and transferred into 1.7 mm NMR tubes. 2D NMR spectra (COSY, TOCSY, J -resolved, HSQC, HMBC) were recorded at 292 K on a Bruker DRX 600 spectrometer equipped with a 3 mm dual inverse cryogenic probe, using standard acquisition parameters. HSQC and HMBC experiments were optimized for $^1J_{\text{C,H}} = 145 \text{ Hz}$ and $^nJ_{\text{C,H}} = 6 \text{ Hz}$, respectively. The spectra were referenced to solvent signals set to $\delta(^1\text{H}) = 2.49$ and $\delta(^{13}\text{C}) = 39.51$ for spectra recorded in DMSO- d_6 , and to $\delta(^1\text{H}) = 3.31$ and $\delta(^{13}\text{C}) = 49.15$ for spectra recorded in methanol- d_4 .

Data reduction and pattern recognition

^1H -NMR spectra were corrected for phase and baseline distortions using a Matlab script, *NMRproc*, developed at Imperial College (T. Ebbels and H. Keun). The spectra were reduced to 244 sequentially integrated spectral regions ("buckets") of 0.04 ppm width between $\delta = 0.22$ and 9.98 using AMIX ver. 3.2.2 software (Bruker BioSpin; Karlsruhe, Germany). In addition, intensity values of all data points in the spectral range from $\delta = 0.50$ to 10.00 were projected on a common chemical shift scale for pattern recognition using spline cubic interpolation; this was carried out using a Matlab script developed by O. Cloarec. Matlab software ver. 7.1 (MathWorks; Natick, MA, USA) was used.

Regions corresponding to the major peak of fatty acids ($\delta = 1.20$ –1.30), residual toluene ($\delta = 2.20$ –2.32 for DMSO- d_6 and $\delta = 2.20$ –2.36 for methanol- d_4 solution, and $\delta = 7.08$ –7.28 for both solvents), residual NMR solvent ($\delta = 2.32$ –2.64 and $\delta = 3.20$ –3.40 for DMSO- d_6 and methanol- d_4 , respectively), and water ($\delta = 3.16$ –3.48 for DMSO- d_6 and $\delta = 4.50$ –5.98 for methanol- d_4) were removed from the data sets. This led to data sets containing 223 or 185 variables for the integrated data, and 33757 or 29483 variables for the full-resolution data, for the spectra acquired in DMSO- d_6 and methanol- d_4 , respectively.

Principal component analysis was performed using SIMCA-P ver. 10.0 software (Umetrics; Umeå, Sweden), using mean-centered scaling.

Results and Discussion

Commercial formulations of St. John's wort were extracted with mixtures of methanol and pyridine, a solvent mixture previously found to enable quantitative extraction (A. G. Jensen and S. H. Hansen, The Danish University of Pharmaceutical Sciences, personal communication). Expansion of ^1H -NMR spectra of the extracts, recorded in $\text{DMSO-}d_6$ and $\text{methanol-}d_4$, are shown in Fig. 1. Fatty acids, flavonol glycosides, chlorogenic acid and signals in the sugar region dominate the spectra. Assignments of the major constituents of the spectra were performed using 2D NMR experiments (COSY, TOCSY, J -resolved, HSQC, and HMBC) with reference to data reported by Bilia et al. [16]. Because of the complexity of the spectra, complete assignment of all resonances was not possible. However, following the principal component analysis (PCA) of the data, identification of quercetin, rutin, hyperoside, and chlorogenic acid was confirmed by spiking with authentic samples.

One of the most common methods of data reduction in NMR spectroscopy for pattern recognition is integration or "bucketing" within narrow sections of the spectral region, typically 0.04 ppm in width [8]. This method is used in order to minimize possible effects of chemical shift changes caused by differences in pH, ionic strength, and specific solute interactions [17]. However, important details may be lost, leading to poorer statistical models. In this work, PCA has been applied to the integrated NMR data sets as well as to the full-resolution data sets of the spectra acquired in $\text{DMSO-}d_6$ and $\text{methanol-}d_4$ in order to compare the two methods. For the integrated data, the resolution was 24 Hz per data point, whereas the resolution of 0.15 Hz per data point was obtained with the full-resolution data sets. To account for possible variations in the total extract concentration of the $\text{methanol-}d_4$ solutions, which were subjected to more extensive manipulations (centrifugation, aliquot transfers) than the $\text{DMSO-}d_6$ solutions, the spectra recorded in $\text{methanol-}d_4$ were normalized either to total integral intensity, or by applying closure of a linearly invariant set of variables. The multivariate NMR spectral data sets can be described as p variables on n observations (the spectra), X_1, X_2, \dots, X_n ; x_{ij} is the j th intensity measure in the i th spectrum. In the latter normalization scheme (H. Keun, Imperial College, personal communication) the variables were not centered, but were scaled by the standard deviation calculated around zero:

$$x_{ij}^{\text{scaled}} = \frac{x_{ij}}{s_{ij}}$$

where s_{ij} is the standard deviation calculated around zero:

$$s_{ij} = \sqrt{\frac{1}{n} \sum_{i=1}^n x_{ij}^2}$$

Thereby, the variables remained in fixed ratios to each other across all samples. The scores of the first PC of the PCA analysis based on the scaled data have been used as the normalization factor, since the largest directly correlating set of variables typically reflects the overall sample concentration:

$$x_{ij}^{\text{normalized}} = \frac{x_{ij}}{t_{[1],i}}$$

where $x_{ij}^{\text{normalized}}$ is the normalized variable and $t_{[1],i}$ is the score value of the first PC for the i th spectrum. A comparison of raw data and data normalized by applying closure of a linearly invariant set of variables is shown for three replicates of preparation 5 in Fig. 2. Since this normalization of spectra acquired in $\text{methanol-}d_4$ was superior to the normalization to total integral intensity of the spectra, it was used in subsequent PCA analysis.

PCA applied to the data sets of integrated data for both solvents showed a tight clustering, with preparations 1 and 9 separated clearly in PC2 and PC1, respectively, from the remaining eight preparations, which were grouped closely together in the two-component PCA model (data not shown). The same pattern was observed when PCA was applied to the full-resolution data sets. In fact, the different appearances of the spectra of extracts of preparations 1 and 9 as compared to the remaining extracts were evident from visual inspection of the spectra. The PCA loadings of the models derived from the full-resolution data sets revealed that a mixture of lactose anomers was responsible for the separation of preparation 9, whereas sucrose accounted for the separate clustering of preparation 1. Pure spectra of these compounds were obtained from the respective PCA loadings. The presence of lactose and sucrose in commercial preparations of St. John's wort can be due to pharmaceutical excipients, and further pattern analysis was therefore performed using higher PCs.

3D score plots of PC3, PC4, and PC5 showed tight clustering of individual preparations according to supplier, both for the integrated data sets and for the full-resolution data sets, especially for the spectra acquired in $\text{DMSO-}d_6$ (Fig. 3). For the full-resolution spectra acquired in $\text{methanol-}d_4$, preparations 3, 4 and 6 are separated from the remaining preparations, which group tightly in the score plot (Fig. 3). The fact that the five-component PCA models derived from the integrated data sets account for 97.9% or 98.6% ($\text{DMSO-}d_6$ and $\text{methanol-}d_4$, respectively) of the variation in the data, whereas the five-component PCA models derived from the full-resolution data sets account for 96.4% or 94.6% ($\text{DMSO-}d_6$ and $\text{methanol-}d_4$, respectively) (Fig. 3), is evidently due to a considerably higher complexity and information richness of the latter data sets. It is noteworthy that preparations 5, 7, and 8 are highly similar, as shown by the integrated data sets for both solvents (Fig. 3). Interestingly, no differentiation of the preparations into classes of tablets and capsules was observed. This suggests that the extracts of St. John's wort used in the manufacturing, and not the manufacturing process itself, are the main source of variability.

In order to identify the secondary metabolites responsible for the observed clustering, each data set was divided into two subsets, containing only tablets or only capsules. When PCA was applied to these data sets, a clear separation of clusters of repeats was observed, both for integrated data sets and for full-resolution data sets (Fig. 4). PCA loadings of the models derived from the full-resolution data sets were used to identify the constituents responsible for the observed clustering.

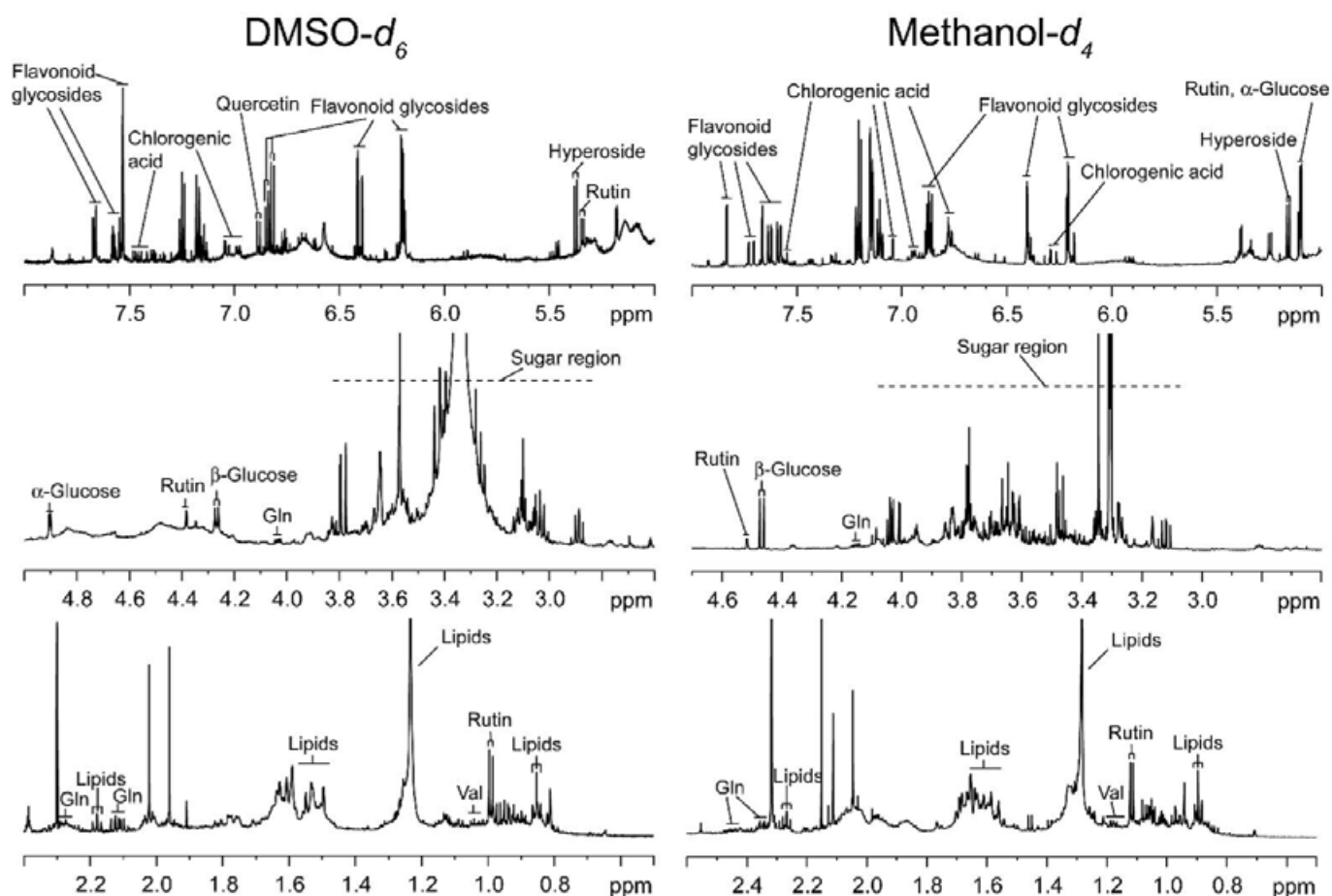


Fig. 1 Expansions of 600 MHz ^1H -NMR spectra of an extract of a commercial formulation (preparation 5) of St. John's wort recorded in $\text{DMSO}-d_6$ (left) and methanol- d_4 (right).

For tablets, score plots derived from integrated data (Fig. 4, left column, top and center) showed a clear discrimination of preparation 3 from the remaining preparations in PC2, whereas preparation 2 grouped separately in PC3. A comparison of the score plot derived from the integrated data set with that derived from the full-resolution data set (methanol- d_4 as solvent) revealed the same tendency in clustering (Fig. 4, left column, center and bottom). However, the discrimination of preparation 4 in PC2 was more obvious in the score plot derived from the full-resolution data, compared to the model based on integrated data, and preparation 2 grouped separately in the other direction in PC3. Analysis of the corresponding loadings revealed that increased content of quercetin and to a lesser extent chlorogenic acid was responsible for the observed separation of preparation 3. The separation of preparation 2 was caused mainly by a higher content of hyperoside and to a lesser extent rutin. The separation of preparation 4 from other tablets was caused mainly by fatty acids, which was also the case when this preparation was analyzed together with all other preparations.

For capsules, the PCA score plots for the integrated data showed the same clustering pattern for both solvents (Fig. 4, right column, top and center). Thus, preparations 5, 7, and 8 were separated from the other preparations in PC2, and preparations 6 and 10 grouped separately in PC3. Score plots for capsules, based on integrated and full-resolution spectra acquired in methanol-

d_4 (Fig. 4, right column, center and bottom), showed the same clustering pattern; however, preparations 6 and 10 grouped in different directions in PC3 in the score plot derived from full-resolution data set. Analysis of the corresponding loadings revealed that increased content of mainly hyperoside, but also of rutin and chlorogenic acid, were responsible for the observed clustering of preparations 5, 7 and 8. Higher content of quercetin, but also of fatty acids, as compared with other preparations formulated as capsules, caused separation of preparation 6. Lower levels of quercetin as compared with other capsules caused the separation of preparation 10 in PC3.

Further analysis of the highly similar preparations 5, 7, and 8 was considered interesting since these preparations originated from the same supplier. Preparations 7 and 8 are different batches of the same brand, and preparation 5 was sold under a different brand name but with the same label insert as the other two. The declared content of the capsules was identical. By applying PCA to preparations 5, 7, and 8 it was not possible to differentiate between the preparations when integrated and full-resolution data from spectra recorded in methanol- d_4 were analyzed (data not shown), but non-overlapping clustering was observed in models derived from full-resolution data acquired in $\text{DMSO}-d_6$ (Fig. 5). Although the differences between these samples are small, interpretation of loadings revealed that flavonoids and to a lesser extent chlorogenic acid account for the observed clustering of pre-

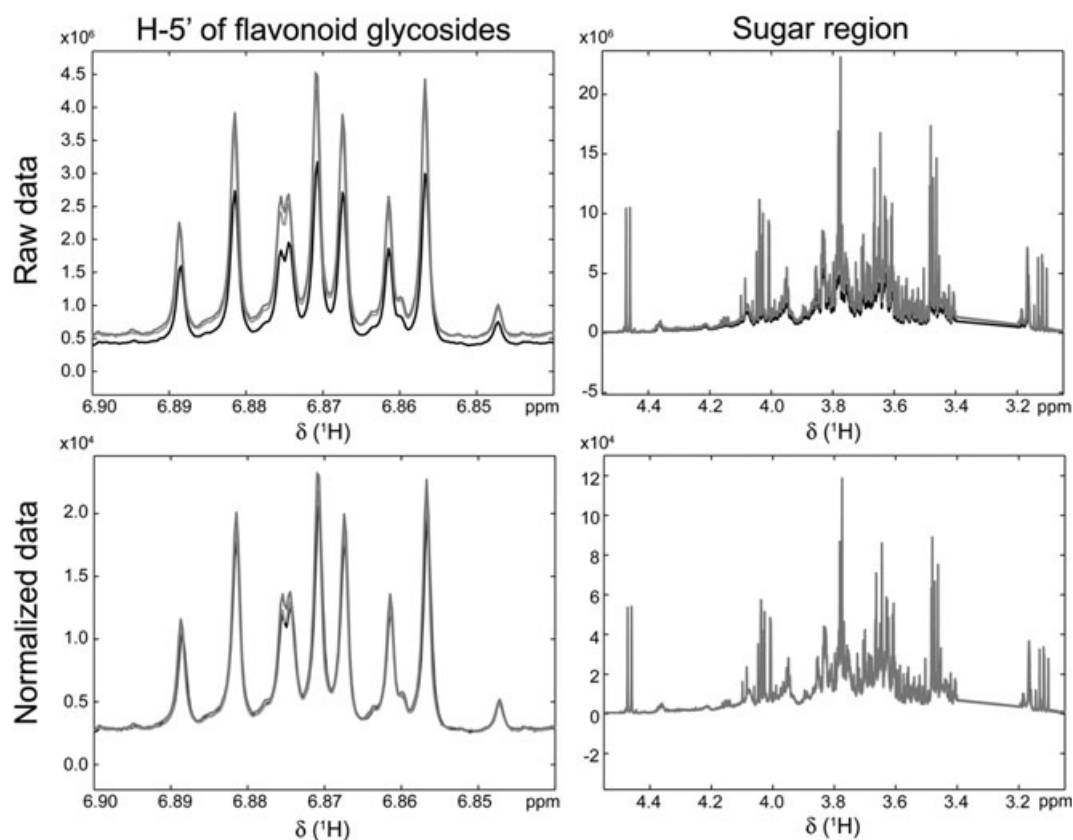


Fig. 2 Comparison of raw data and data normalized by applying closure of a linearly invariant set of variables. Selected regions of the ^1H -NMR spectra of three replicates of the extract of preparation 5 recorded in methanol- d_4 are shown. *Left*: signals of H-5' of flavonoid glycosides. *Right*: sugar region of the spectra.

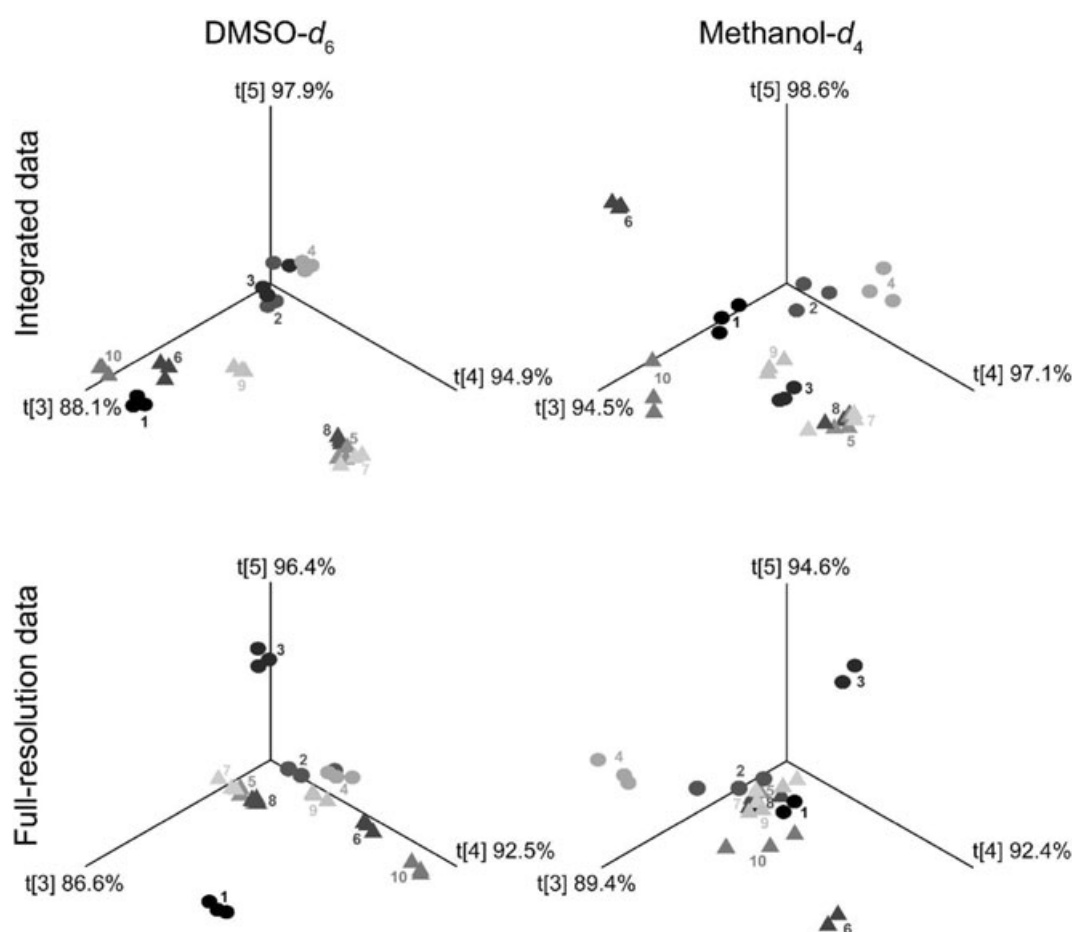


Fig. 3 3D score plots (PC3-PC5) for DMSO- d_6 and methanol- d_4 solutions of extracts of preparations 1–10 of St. John's wort. *Top*: models derived from integrated data sets. *Bottom*: models derived from full-resolution data sets. Tablets are marked with circles and capsules with triangles.

paration 7, whereas higher levels of fatty acids in preparation 5 compared with that in preparation 8 had an influence on their separate clustering.

In terms of detection of different levels of individual extract constituents, the use of full-resolution data sets gave particularly valuable information. This is illustrated in Fig. 6, where loadings for the integrated and full-resolution data sets, corresponding to PC3 in the score plots for tablets in methanol- d_4 (Fig. 4, left column, center and bottom), are compared. The loadings from the full-resolution data set have been multiplied by -1 to compensate for the grouping of preparations in different directions in PC3 in the score plots. It is apparent that the ^1H -NMR signals of closely related compounds, in this case quercetin, rutin and hyperoside, can appear in the same “bucket”, leading to loss of important information about the content of individual constituents. Negative loading values (weights) for H-5' and H-8 of quercetin and positive loadings for H-5' and H-8 of hyperoside and rutin are clearly apparent in the loading line plot for the full-resolution data (Fig. 6), demonstrating differences between the preparations at the individual compound level. Furthermore, the positive loading for the bucket 6.18–6.22 ppm is made up from the contributions from rutin and hyperoside, which is only

disclosed by the full-resolution data. Because “buckets” typically contain resonances from several compounds, such differentiation is not possible for integrated data. The use of full-resolution ^1H -NMR data is thus especially important for flavonoids and flavonoid glycosides, as only small chemical shift differences are observed for the flavonoid moiety. The substantial differences observed in the sugar region of the ^1H -NMR spectra of flavonoid glycosides are for practical purposes excluded from the interpretation of loadings.

No clustering of data attributable to differences in the content of hypericins or hyperforins was observed in this study. While the content of hypericins in the commercial preparations is standardized and expected to be fairly constant, the level of hyperforins is low and no ^1H -NMR signals attributable to these constituents (hyperforin, adhyperforin) are immediately recognizable (Fig. 1). The major variations in the composition of the preparations are due to the content of flavonoids, which are definitely linked to the pharmacological activity of St. John's wort [18], [19], [20], [21].

In conclusion, this study has shown the effectiveness of the use of ^1H -NMR spectra combined with PCA for comparison of sam-

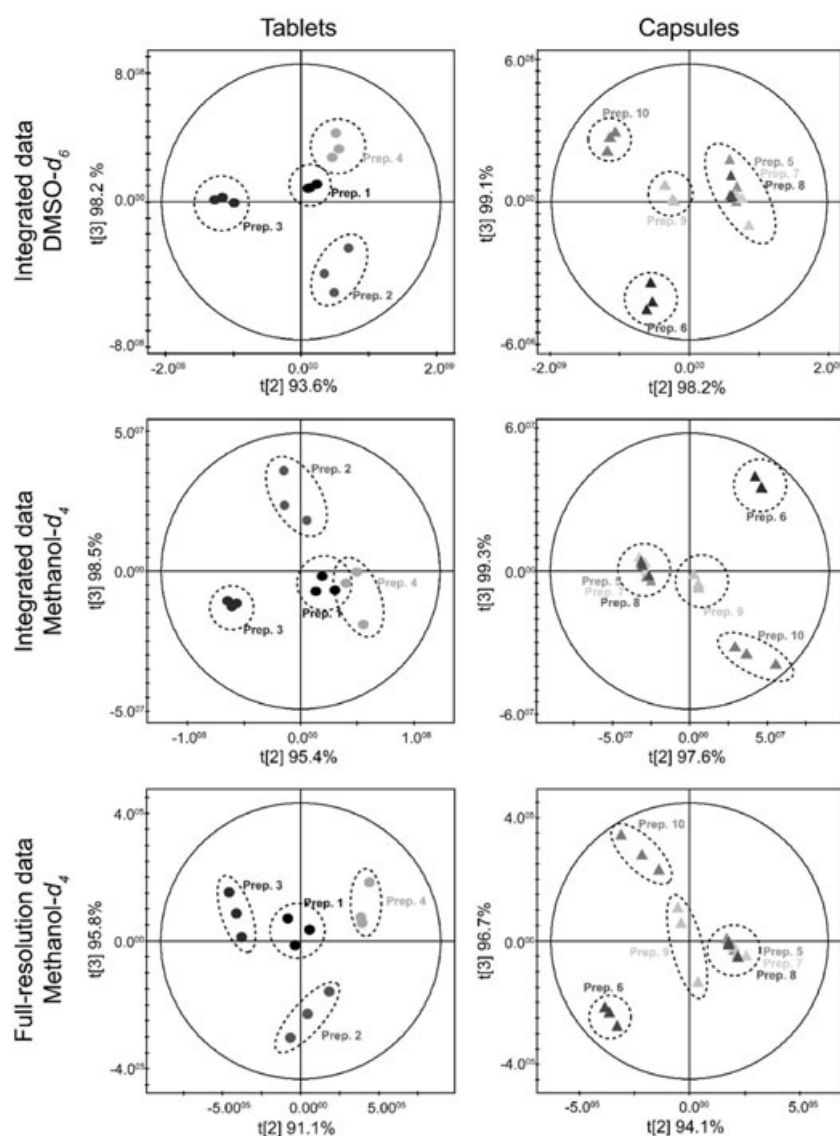


Fig. 4 Score plots for ^1H -NMR data for tablets and capsules extracts. *Top*: integrated data sets, spectra acquired in $\text{DMSO-}d_6$. *Center*: integrated data sets, spectra acquired in $\text{methanol-}d_4$. *Bottom*: full-resolution data sets, spectra acquired in $\text{methanol-}d_4$.

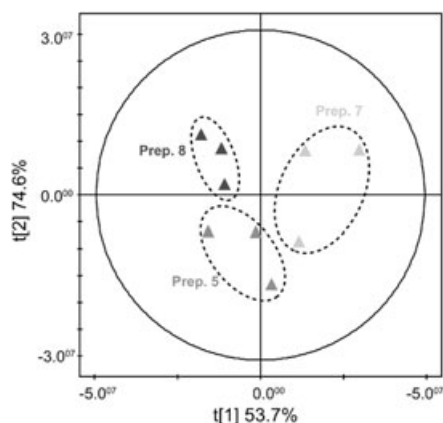


Fig. 5 Score plot for preparations 5, 7, and 8 (capsules with the same label insert and identical declared content) derived from full-resolution data sets acquired in DMSO- d_6 .

ples of commercial preparations of St. John's wort. Although the samples have been subjected to the same standardization procedure [1], the analysis disclosed considerable differences in the product composition including discrimination between samples from different suppliers, but also between different batches of the same product from the same supplier. Because of the uncertainties regarding the compounds responsible for pharmacological activity of extracts of St. John's wort, these differences may cause different quality of the products in terms of their action, and may explain ambiguous results often shown in clinical trials with herbal medicines. Thus, the results of this study further emphasize the value of PCA of ^1H -NMR spectral data as a sensitive method for characterization of very complex pharmaceutical preparations, which takes all detectable constituents into account in the description of sample composition. Moreover, the work demonstrates benefits from the use of two different NMR solvents, which give complementary PCA models with different information content.

In this work, use of full-resolution ^1H -NMR data was applied for the first time for the characterization of plant extracts or herbal medicines, using about 30 000 variables for PCA analysis, as

compared to a few hundred variables that are available when integrated data are used. Although clustering observed in the models based on integrated and full-resolution data sets was generally the same, only the use of full-resolution data allowed identification of individual, closely related extract constituents responsible for differentiation in the PCA models. Since plants often contain series of closely related metabolites, we conclude that the use of full-resolution data is preferable for construction of chemometric models based on ^1H -NMR spectra of extracts of plants and herbal medicines.

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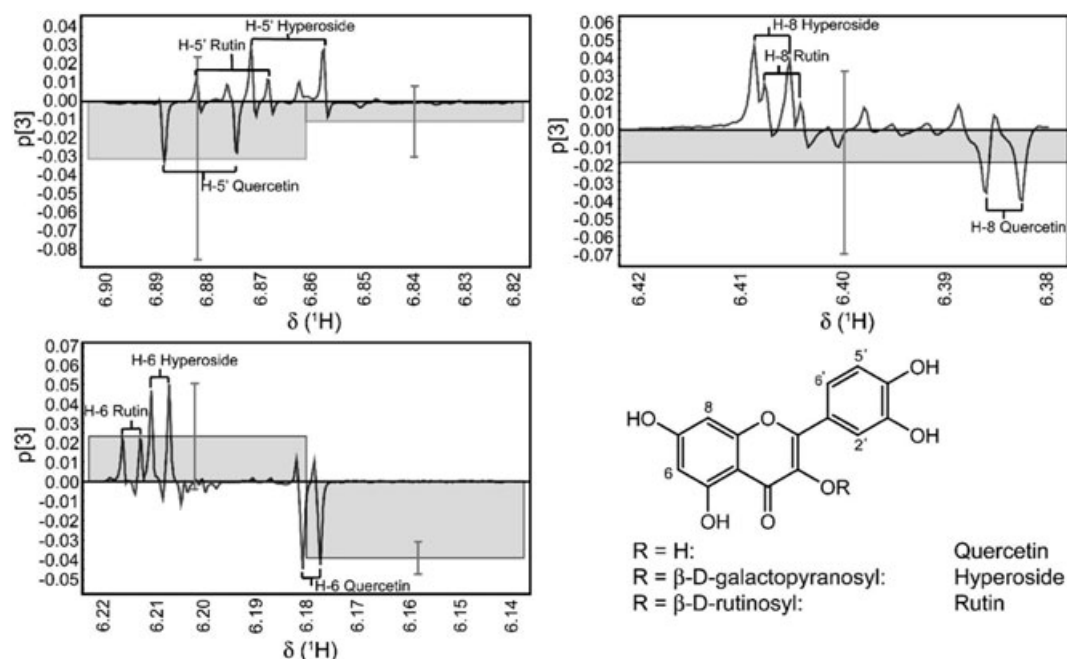


Fig. 6 Loadings corresponding to PC3 in score plots of tablet extracts shown in Fig. 4 in methanol- d_4 , for models based on integrated data (shaded bars) and full-resolution data (solid lines). Chemical shift regions of H-5' (top left), H-8 (top right) and H-6 (bottom left) of closely related flavonoids are shown.

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